MUSCARINIC RECEPTOR IN *DROSOPHILA MELANOGASTER* DEMONSTRATED BY BINDING OF [^{3}H]QUINUCLIDINYL BENZILATE

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1. Introduction

Cholinergic receptors in vertebrates are usually classified into two main types, nicotinic and muscarinic [1]. It has been suggested that cholinergic receptors in insects do not fall into one of these categories but are of a mixed nicotinic and muscarinic nature [2–5]. However, it was recently found that flies do contain a nicotinic receptor similar to that of vertebrates ([6–9], Rudloff, submitted). We report here that the fruit fly, *Drosophila melanogaster,* contains also a distinct muscarinic receptor, as revealed by specific binding of the powerful muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) [10]. It appears that in contrast with vertebrate brain, fly head is richer in nicotinic receptors than in muscarinic receptors.

2. Materials and methods

Drosophila melanogaster, Canton-Special strain, were cultured under standard conditions [11]. Experiments were performed on isolated heads. They were separated from bodies by freezing and shaking and homogenized (400–500 heads/ml) in 0.32 M sucrose, in a glass–glass dual homogenizer followed by a glass–Teflon homogenizer. The supernatant of 500 × *g* centrifugation for 10 min was used. [3-^{3}H]Quinuclidinyl benzilate (8.4 Ci/mmol) was from The Radiochemical Centre, Amersham. Dexetimide was a generous gift of Janssen Pharmaceutica, Beerse, Belgium. α-Bungarotoxin (α-Btx) was purified from crude *Bungarus multicintus* venom [12]. Other chemicals were of analytical grade.

Binding of [^{3}H]QNB was assayed as follows: Aliquots of fly homogenate (containing up to 0.7 mg protein) were incubated at 25°C in 0.06 M NaCl, 1 mg/ml BSA, 0.025 M Tris–HCl, pH 7.4 (Buffer I), in total vol. 0.25 ml. Reaction was started by addition of the appropriate concentration of [^{3}H]QNB and was terminated by diluting with 2 ml buffer I followed immediately by vacuum filtration through a glass-fiber filter (GF/C, Tamar, Israel). The filter was then washed 3 times with 2 ml portions of buffer I, dried and placed in vials containing 4 ml 33% (v/v) Triton X-100, 0.8% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP) in toluene. Vials were maintained for 12–24 h at 25°C and counted by liquid scintillation spectrometry.

3. Results and discussion

Under the conditions employed, binding of [^{3}H]QNB was linearly proportional to the amount of head homogenate present. Specific binding, defined as total binding minus binding occurring in the presence of 0.1 mM atropine, was saturable (fig.1). After incubation for 1 h, maximum specific binding was obtained with [^{3}H]QNB concentrations higher than 5 nM. Non-specific binding at that concentration was <5% of total. Half-saturation occurs at about 2 nM. Concentration of binding sites in head homogenate was found to be 0.08 ± 0.03 pmol/mg protein.

The time dependence of binding is described in fig.2. Under the conditions employed, binding reached half-maximal values in about 5 min and maximal values in about 40 min. The on-rate constant for the formation of [^{3}H]QNB–receptor complex was

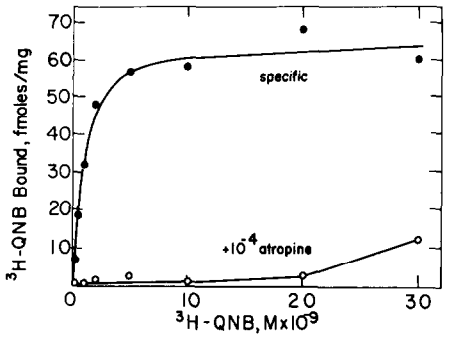

Fig.1. [^{3}H]QNB binding to *Drosophila* head homogenate after incubation for 1 h at various [^{3}H]QNB concentrations. Specific binding is defined as total binding minus binding in the presence of 10^{-4} M atropine.

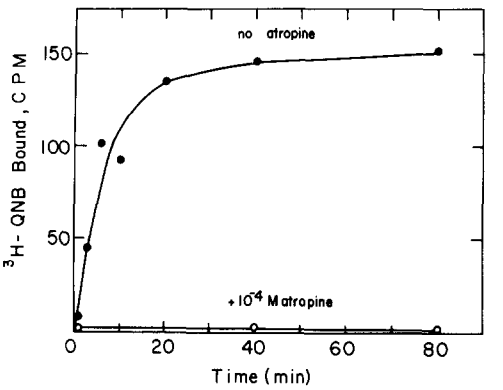

Fig.2. Time dependence of [^{3}H]QNB binding to *Drosophila* head homogenate. Incubation medium contained 5 nM [^{3}H]QNB and 0.7 mg head protein.

calculated to be K_1 1.5×10^5 $M^{-1}.s^{-1}$, assuming a simple bimolecular reaction between ligand and receptor.

Specificity of binding and pharmacological properties were studied by testing the effect of various cholinergic ligands on binding. Concentrations of various drugs that displaced binding by 50% (ED_{50}) are given in table 1. The muscarinic ligands dexetimide, scopolamine and atropine were most powerful in protecting against [^{3}H]QNB binding. D,L-Muscarine, which has no effect on binding of α-Btx to fly nicotinic receptors at concentrations up to 1 mM [7], inhibits [^{3}H]QNB binding with ED_{50} 6×10^{-5} M. Acetylcholine is also effective in protecting [^{3}H]QNB binding-sites (ED_{50} 3×10^{-5} M). Nicotine, which has high affinity for fly cholinergic nicotinic receptor [7], is not effective in protecting [^{3}H]QNB binding-sites at concentrations up to 1 mM. α-Btx had no significant effect on [^{3}H]QNB binding at 10 μM.

Table 1
Effect of various cholinergic ligands on [^{3}H]QNB-binding to *Drosophila* head homogenate

Ligand	ED_{50}
Dexetimide	4×10^{-9} M
Scopolamine	7×10^{-9} M
Atropine	3×10^{-8} M
Acetylcholine[a]	3×10^{-5} M
Pilocarpine	3×10^{-5} M
D-Tubocurarine	4×10^{-5} M
D,L-Muscarine	6×10^{-5} M
Decamethonium[b]	$> 10^{-3}$ M
Nicotine[b]	$> 10^{-3}$ M

[a]Determined in the presence of 10^{-5} M diethylfluorophosphate, which completely inhibits acetylcholinesterase
[b]Tested up to a concentration of 10^{-3} M

Aliquots containing 0.7 mg head protein were preincubated for 25 min with the appropriate concentration of ligand, except for acetylcholine, which was preincubated for 15 min Reaction was started by addition of [^{3}H]QNB (5 nM) and was carried out for 60 min at 25°C. ED_{50} was determined from plots of relative [^{3}H]QNB binding versus ligand concentration.

The above data clearly indicate that *Drosophila* contain a muscarinic cholinergic receptor, as revealed by [^{3}H]QNB binding. Pharmacological properties of that receptor are similar to those reported for mammalian muscarinic receptor [10]. However, whereas mammalian brain is an order of magnitude richer in muscarinic receptors than in nicotinic receptors [10,13], in *Drosophila* head concentration of the nicotinic receptor (>0.4 pmol/mg, [8]) is higher than that of the muscarinic receptor. It should be noted that our assay reveals only binding to particulate muscarinic receptors, and additional, soluble [^{3}H]QNB-binding components cannot be excluded.

The observation that mammals and insects, which are far remote on the phylogenetic scale, both contain distinct cholinergic muscarinic and nicotinic receptors, indicates that both types of cholinergic receptor specificities evolved early during evolution. It is possible that invertebrates contain additional

types of cholinergic receptors. It has been reported, on the basis of electrophysiological and pharmacological studies, that molluscan neurons contain three types of cholinergic receptors, two of which are nicotinic, the third displaying neither a classical nicotinic nor a classical muscarinic nature [14]. Jewess et al. [15] reported that high-speed supernatant of house-fly heads homogenate contains a cholinergic receptor with a mixed nicotinic and muscarinic nature, that has high affinity for muscarinic ligands, e.g., dexetimide and atropine, and for nicotinic ligands, e.g., nicotine and decamethonium. The preparation also binds QNB (cited as personal communication, [9]). It is of interest to note that neither the nicotinic receptor of *Drosophila* [7,9] nor the muscarinic receptor reported here, display high affinity for decamethonium. The decamethonium binding component may therefore represent an additional type of cholinergic receptor. Further biochemical studies, combined with genetic dissection methods for which *Drosophila* is particularly suitable [16], may shed light on the molecular and physiological characteristics of various cholinergic receptors in flies. One should also note that further characterization of cholinergic receptors in insects may lead to developing more effective insecticides [17].

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